Cisplatin enhances the anticancer effect of β-lapachone by upregulating NQO1

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NAD(P)H:quinone oxidoreductase (NQO1) has been reported to play an important role in cell death caused by β-lapachone (β-lap), 3,4-dihydro-22,2-dimethyl-2H-naphthol[1,22b]pyran-5,6-dione. This study investigated whether cisplatin (*cis*-diamminedichloroplatinum) sensitizes cancer cells to β-lap by upregulating NQO1. The cytotoxicity of cisplatin and β-lap alone or in combination against FSaII fibrosarcoma cells of C3H mice *in vitro* was determined with a clonogenic survival assay and assessment of γ-H2AX foci formation, a hallmark of DNA double-strand breaks. The cellular sensitivity to β-lap progressively increased during the 24 h after cisplatin treatment. The expression and enzymatic activity of NQO1 also increased during the 24 h after cisplatin treatment, and dicoumarol, an inhibitor of NQO1, was found to nullify the cisplatin-induced increase in β-lap sensitivity. The role of NQO1 in the cell death caused by β-lap alone or in combination with cisplatin was further elucidated using NQO1-positive and NQO1-negative MDA-MB-231 human breast cancer cells. Cisplatin increased the sensitivity of the NQO1-positive but not the NQO1-negative MDA-MB-231 cells to β-lap treatment. Combined treatment with cisplatin and β-lap suppressed the growth of FSaII tumors in the legs of C3H mice in a manner greater than additive. It is concluded that cisplatin markedly increases the sensitivity of cancer to β-lap *in vitro* and *in vivo* by upregulating NQO1. *Anti-Cancer Drugs* 20:901–909 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Beta-lapachone (3,4-dihydro-*22*,*2*-dimethyl-2H-naphthol [1,22b]pyran-5,6-dione; β-lap) is a quinone-containing anticancer drug originally obtained from lapacho trees in South America [1]. Beta-lap used in combination with other chemotherapy drugs, such as gemcitabine, is currently in phase I/II clinical trials for various human cancers [2]. It was previously reported that β-lap induces apoptosis by activating G_1 or S cell cycle checkpoints, thereby inhibiting the cell cycle progression and causing apoptosis preferentially in transformed cells over normal cells [3–8]. However, other investigators attributed the cell death caused by β-lap to futile cycling between the oxidized and two-electron-reduced forms of hydroquinone, mediated by NAD(P)H:quinone oxidoreductase (NQO1), resulting in generation of reactive oxygen species [9–13]. Indications are that two-electron-reduced β-lap is oxidized first to one-electron-reduced semiquinone, which then causes redox cycling and generation of reactive oxygen species, triggering cascades of molecular changes leading to DNA double-strand breaks and p53-independent apoptosis. The major molecular changes that have been observed in cells treated with β-lap and thus involved in the cell death include depletion of NAD^+ and ATP, loss of mitochondrial membrane potential, loss of Ca^{2+} homeostasis, inhibition of NF-κB, activation of u-calpain, Ca^{2+}-dependent PARP-1 hyperactivation, and degradation of p53 [9–13].

NQO1 is a cytosolic flavoenzyme, which was earlier known as DT-diaphorase (EC 1.6.99.2). NQO1 catalyzes the two-electron reduction of endogenous and exogenous quinone compounds to their corresponding hydroquinone forms using H^+ from NADH and NADPH [14–20]. Interestingly, the two-electron reduction of quinones through NQO1 mediation causes either chemoprotection and detoxification or a chemotherapeutic effect, depending on the target quinones. With respect to chemoprotection and detoxification effects, the toxic quinones are two-electron reduced, conjugated with glutathione, glucuronic acid or other moieties, and excreted from the cells [15,16,21]. In contrast, the NQO1-mediated two-electron reduction of certain quinone compounds, such as β-lap and mitomycin C (MMC) [14–18,22–24], or its analogs, such as EO9 [14,19,20,25,26], triggers a series of cytotoxic signal transduction pathways leading to cell

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death. In this regard, it has been clearly shown that the sensitivity of cells to bioreductive anticancer drugs, such as MMC [22–25] and β-lap [9–13,27–31], is directly related to the cellular NQO1 level.

Importantly, NQO1 activity in many tumors is significantly higher than that in adjacent normal tissues, indicating that tumors may be preferentially damaged by NQO1-dependent anticancer drugs [23,32–34]. The expression of the NQO1 gene is mainly regulated by the antioxidant response element found in the cis-element gene promoter located in the human chromosome 16q22 [35–37]. The NQO1 gene has been shown to be activated by oxidative stress, antioxidants, xenobiotics, ionizing radiation, heat shock, and hypoxia [22,25,27–31,34,38–42]. It has been reported earlier that ionizing radiation with clinically relevant doses, that is 2–4 Gy [27,28,30], or heating at 41–42°C for 1 h [29,31] increased NQO1 levels in various animal and human tumor cells, and sensitized the cells to β-lap treatment *in vitro* and *in vivo*. These observations lead to the question of whether chemotherapy drugs would also upregulate NQO1 in cancer cells and sensitize the cancer cells to β-lap. Cisplatin (*cis*-diamminedichloroplatinum) is an inorganic, square-planar coordination complex that is formed by an atom of platinum surrounded by chlorine and ammonium ions in the *cis*-position of the horizontal plane [43–46]. Cisplatin exerts its anticancer effect by forming covalent binding between the central platinum atom and DNA, resulting in DNA damage and apoptosis [43–46]. The purpose of this study was to test the hypothesis that cisplatin-induced DNA damage might provoke upregulation of NQO1 in cancer cells. It was observed that cisplatin significantly upregulates NQO1 in cancer cells, thereby markedly increasing the sensitivity of the cancer cells to β-lap *in vitro* as well as *in vivo*.

Materials and methods

Cells and cell culture

The cell lines used were the early generation of FSaII fibrosarcoma cells of C3H mice [47] and NQO1-deficient MDA-MB-231 (NQO1-negative) and NQO1-transfected MDA-MB-231 (NQO1-positive) human breast cancer cells [11] obtained from Dr David Boothman, University of Texas Southwestern Medical Center, Dallas, Texas, USA. The cells were cultured in RPMI 1640 medium (Gibco BRI, Grand Island, New York, USA) supplemented with 10% bovine calf serum (Hyclone Laboratories Inc., Logan, Utah, USA), penicillin (50 units/ml), and streptomycin (50 μg/ml) in a 37°C incubator under a mixture of 95% air and 5% CO_2.

Beta-lapachone and cisplatin

Beta-lap and cisplatin were purchased from the Sigma Chemical Co. (St Louis, Missouri, USA). Stock β-lap solution was prepared by dissolving β-lap powder in dimethyl sulfoxide at 5 mmol/l and stored the resultant solution at −20°C. Immediately before use, the stock β-lap solution was diluted to desired concentrations in RPMI 1640 medium for in-vitro study or in HP-β-CD (β-hydroxypropyl-β-cyclodextrin) [48] for in-vivo study. Cisplatin was diluted to desired concentrations in RPMI 1640 medium for in-vitro study and in physiological saline for in-vivo study.

Clonogenic cell death

Cells in exponential growth phase in culture were dispersed to single cells by treatment with a 0.25% trypsin solution containing 1 mmol/l EDTA for 10 min, and then washed twice with a medium containing 10% bovine calf serum. Appropriate numbers of cells were seeded in 25 cm^2 plastic T-25 type tissue culture flasks with 5 ml complete RPMI 1640 medium. After overnight incubation at 37°C in a humidified 95% air–5% CO_2 atmosphere, the cells were treated with β-lap and cisplatin alone or in combination, gently washed twice with medium, and FSaII cells were cultured in complete RPMI 1640 medium for 7–8 days, MDA-MB-231 cells for 8–10 days. The number of colonies was counted, and the surviving cell fractions were calculated.

Gamma-H2AX foci formation

DNA double-strand breaks caused by cisplatin and β-lap alone or their combination were studied using the γ-H2AX foci formation method. FSaII tumor cells were plated on tissue culture chamber slides, incubated at 37°C overnight, and subjected to one of the following four treatments: (i) treated with 5 μmol/l cisplatin for 1 h, (ii) treated with 5 μmol/l β-lap for 1 h, (iii) treated with 5 μmol/l cisplatin and 5 μmol/l β-lap together for 1 h, or (iv) treated with 5 μmol/l cisplatin for 1 h, incubated in regular medium at 37°C for 24 h and then treated with 5 μmol/l β-lap. The treated cells were washed twice with PBS, fixed with a mixture of acetone and methanol (1 : 1) for 20 min, and incubated with anti-γ-H2AX antibody (Millipore Co., Billerica, Massachusetts, USA) (1 : 300 dilution in 1% bovine serum albumin) for 1 h, followed by an incubation with a secondary antibody conjugated with FITC (Invitrogen Co., Carlsbad, California, USA) for 1 h. After being washed three times with PBS, cells were mounted with a mixture of anti-fade reagent and 4′,6′-diamidine-2-phenylindole (DAPI) (Invitrogen Co.). The γ-H2AX foci were examined with a confocal fluorescence microscope.

Immunofluorescence microscopy study for NQO1 expression

The effect of cisplatin treatment on the NQO1 level in FSaII cells was studied using immunofluorescence microscopy. Cells in exponential growth phase in tissue culture chamber slides were treated with 5 μmol/l cisplatin for 1 h at 37°C, washed, further incubated for varying periods in complete medium at 37°C, and washed with

PBS. After fixing with a mixture of acetone and methanol (1 : 1) for 20 min, cells were treated with 1% bovine serum albumin, incubated with anti-NQO1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) (1 : 100 dilution in PBS) for 2 h and further incubated for 1 h with a secondary antibody conjugated with FITC (Invitrogen Co.). The cells were then mounted with 10 µl of Prolong gold antifade reagent with DAPI (Invitrogen Co.), and examined for NQO1 expression with a confocal laser scanning fluorescence microscope.

Western blot analysis for NQO1 expression

The effect of cisplatin on the expression of NQO1 was studied using the western blot method. Cells were exposed to 5 µmol/l cisplatin for 1 h, washed twice with medium, and incubated in complete RPMI 1640 medium for varying lengths of time at 37°C. The cells were harvested by treatment with 0.25% trypsin solution containing 1 mmol/l EDTA for 10 min, washed twice with ice-cold PBS, and dissolved in a solubilizing buffer (pH 7.4, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 20 mmol/l Tris–HCl, 150 mmol/l sodium fluoride, 2 mmol/l phenylmethyl sulfonyl fluoride, 10 mmol/l iodoacetamide, 10 µg/ml aprotinin, and 10 µg/ml leupeptin). Aliquots containing 60 µg of protein were mounted on 12% sodium dodecyl sulfate–polyacrylamide gel and electrophoresed. The polypeptides separated in the gel were transblotted onto Hybond-P (Amersham Life Sciences Inc., Arlington Heights, Illinois, USA) in a transfer buffer (192 mmol/l glycine, 25 mmol/l sodium dodecyl sulfate, and 10% methanol). Blots were blocked with 3% nonfat dry milk in pH 7.4 TBST, incubated with anti-NQO1 antibody (1 : 1000 dilution) (Santa Cruz Biotechnology Inc.) and treated with horseradish peroxides-conjugated anti-rabbit immunoglobulin G secondary antibody (1 : 1000 dilution, Amersham Life Sciences Inc.). The immunoreactive bands were visualized using chemiluminescence. The density of the blots was determined with the Image J computer program (National Institutes of Health, Bethesda, Maryland, USA). Equal sample loading was confirmed by reprobing the same blots with mouse monoclonal antiserum against β-tubulin.

Determination of NQO1 activity

NQO1 activity in the cells was measured by spectrophotometry using 2, 6-dichlorophenolindophenol (DCPIP) as substrate [23]. FSaII cells were treated with 5 µmol/l cisplatin for 1 h at 37°C, washed twice with medium, and incubated in complete RPMI 1640 medium for 24 h. After washing twice with ice-cold PBS, cells were harvested with ice-cold TE buffer (pH 7.4 50 mmol/l Tris, 7.4 µg/ml EDTA, 10 µg/µl aprotinin), and then sonicated and centrifuged at 13000 rpm for 30 min. The supernatants were collected in ice-cold tubes, mixed with the reaction reagent (25 mmol/l pH 7.4 Tris, 200 µmol/l NADH, 0.07% bovine serum albumin (w/v), 40 µmol/l DCPIP), and the absorbance was measured at 600 nm for 2 min in the presence or absence of 20 µmol/l dicoumarol (NQO1 inhibitor). The results were calibrated as reduced µmol DCPIP/min/mg protein using an extinction coefficient of 21.1 m/mol/l/cm.

The role of NQO1 in cell death caused by β-lap alone or in combination with cisplatin

Dicoumarol [3,3′methylene-bis (4-hydroxycoumarin)], an inhibitor of NQO1, has been shown to suppress β-lap-induced clonogenic cell death, indicating that NQO1 plays an important role in β-lap-induced cell death [27–31]. In this study, it was investigated whether NQO1 was involved in the cell death caused by the combination of cisplatin and β-lap. Cells were treated with 5 µmol/l cisplatin for 1 h, rinsed twice with medium, and further incubated for 24 h in complete medium at 37°C. The cells were then incubated with 5 µmol/l β-lap with or without 50 µmol/l dicoumarol (Sigma-Aldrich Co., St Louis, Missouri, USA) for 4 h, washed, and clonogenic survival was determined. As the control, the effects of dicoumarol on the clonogenic cell death caused by cisplatin or β-lap alone were also investigated.

The role of NQO1 in the cell death caused by β-lap alone or in combination with cisplatin was further studied with NQO1-positive and NQO1-negative MDA-MB-231 human breast cancer cells. Cells were treated with 5 µmol/l β-lap for 1 h or pretreated with 5 µmol/l cisplatin for 1 h and treated 24 h later with 5 µmol/l β-lap for 1 h, and the clonogenic survival of the cells was obtained.

Tumor growth delay

FSaII cells in exponential growth phase in culture were harvested by trypsin treatment, washed twice in medium, and suspended in serum-free medium. Approximately, 5×10^5 cells in 0.05 ml serum-free medium were injected subcutaneously into the hind legs of female C3H mice weighing about 20 g each. When tumors grew to 90–110 mm^3 in 7–9 days, the host mice were randomly divided into four groups. Cisplatin was dissolved in physiological saline and β-lap was dissolved in HP-β-CD solution immediately before injection. A group of mice was treated with a combination of cisplatin and β-lap by injecting intraperitoneally (i.p.) with 2 mg/kg cisplatin on days 1, 3, 5, and 7, and 40 mg/kg β-lap on days 2, 4, 6, and 8. Other groups of mice were treated with cisplatin alone or β-lap alone every other day for four total treatments. The concentrations of the drugs were adjusted to allow an i.p. injection of about 0.1 ml of the drug solutions each time. The shortest and longest diameters of each tumor were measured with a caliper, and β-lap tumor volumes were calculated with the formula $V = a^2b/2$, where a and b are the shortest and longest diameters, respectively [27,29,30]. Animal experiments were performed following a protocol (Protocol number: 0412A66350) approved by the University of Minnesota Institutional Animal Care and Use Committee.

Results

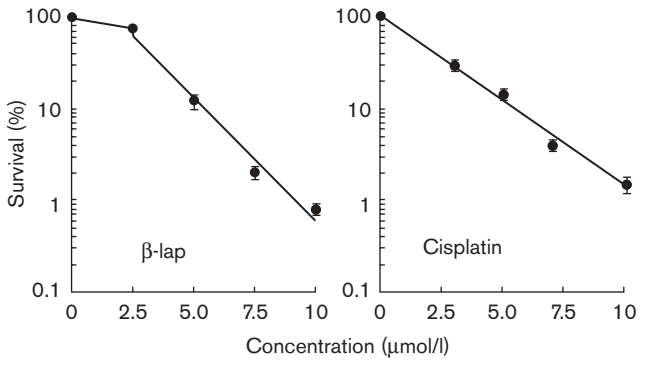
The effects of β-lap and cisplatin on the clonogenic survival of FSaII tumor cells

The effects of 4-h incubation with different concentrations of β-lap or 1 h incubation with different concentrations of cisplatin on the clonogenic survival of FSII tumor cells are shown in Fig. 1. The clonogenic cell survival decreased slightly after treatment with 2.5 μmol/l β-lap, but it decreased exponentially as the concentration of β-lap was increased. The clonogenic survival of cells treated with cisplatin for 1 h exponentially declined as a function of the drug concentration.

The combined effects of β-lap and cisplatin on clonogenic cell survival

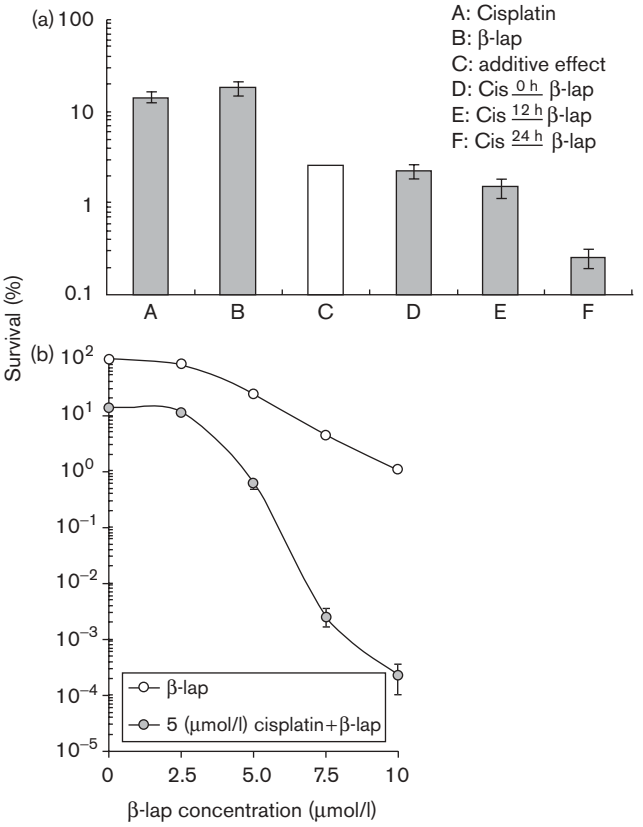
The effects of a combination of cisplatin and β-lap treatment on the clonogenic survival of FSaII cells are shown in Fig. 2a. A 1-h treatment with 5 μmol/l cisplatin alone decreased the cell survival to 14.0 ± 1.5%, and a 4-h treatment with 5 μmol/l β-lap decreased the cell survival to 18.1 ± 2.9%. When cells were treated first with 5 μmol/l cisplatin for 1 h, and then washed and immediately retreated with 5 μmol/l β-lap, the cell survival declined to 2.1 ± 0.5%. The treatment of cells for 4 h with 5 μmol/l β-lap at 12 and 24 h after cisplatin treatment reduced the clonogenic cell survival to 1.4 ± 0.7 and 0.24 ± 0.5%, respectively. If the combination of 5 μmol/l cisplatin and 5 μmol/l β-lap reduced cell survival in an additive manner, 2.5% (i.e. $0.14 \times 0.18 = 0.025$) of cells would survive (open column in Fig. 2a). Therefore, it may be concluded that the cell death caused by the cisplatin treatment immediately followed by β-lap treatment (i.e. 2.1% survival) was additive, whereas the cell death caused by the combination of cisplatin pretreatment and β-lap treatment separated by a 12–24 h interval, particularly a 24-h interval (0.24% survival), was apparently greater than additive. Figure 2b shows the clonogenic survival of FSaII cells treated with graded doses of β-lap alone (open circle) for 4 h and the survival of cells that were pretreated with 5 μmol/l cisplatin for 1 h and then treated 24 h later with graded doses of β-lap for 4 h (closed circle). As the β-lap concentration was increased above 2.5 μmol/l, the cell survival curve for the combined treatment became markedly steeper relative to the survival curve for the β-lap treatment alone. It may be concluded that pretreatment with cisplatin markedly increased the sensitivity of the cells to β-lap treatment applied 24 h later.

Fig. 1



The percentage survival of FSaII tumor cells treated with β-lapachone (β-lap) or cisplatin. Cells were incubated with different concentrations of β-lap for 4 h or cisplatin for 1 h at 37°C, washed, and then cultured for 7–8 days under the standard culture condition. The averages of six to eight experiments with duplicate cultures ± 1 SE are shown.

Fig. 2



(a) Combined effects of cisplatin and β-lapachone (β-lap) on the percentage of clonogenic survival of FSaII tumor cells. As controls, cells were treated with 5 μmol/l cisplatin for 1 h alone (A) or 5 μmol/l β-lap for 4 h alone (B), washed, incubated for 7–8 days, and then the clonogenic survival was determined. For the combined effects, cells were treated with 5 μmol/l cisplatin for 1 h, washed, incubated for 0, 12, or 24 h (D–F), and then treated with 5 μmol/l β-lap for 4 h, washed, and then the clonogenic survival was determined by culturing the cells for 7–8 days. The averages of eight experiments ± 1 SE are shown. The open column (C) indicates the hypothetical survival rate of FSaII tumor cells if the combined effects of cisplatin and β-lap on the cell survival were additive. (b) Survival curves of FSaII tumor cells treated with β-lap alone or in combination with cisplatin. Cells were treated with 5 μmol/l cisplatin for 1 h, rinsed, incubated for 24 h, and then treated with varying concentrations of β-lap for 4 h (closed circles). As the control, cells were treated with β-lap without pretreatment with cisplatin (open circles). After the treatments, cells were washed, incubated and the percentages of clonogenic cell survivals were obtained. The averages of seven to nine experiments with duplicate cultures ± 1 SE are shown.

Gamma-H2AX foci formation

Figure 3a shows the γ-H2AX foci, a hallmark of DNA double-strand breaks, in FSaII tumor cells treated with cisplatin alone, β-lap alone, or a combination of the two treatments. In the untreated control cells, the number of γ-H2AX foci observed in each cell varied between 2 and 7. Therefore, the cells showing more than seven foci were counted as γ-H2AX-positive cells. From the number of γ-H2AX-positive cells and the total number of cells, that is, DAPI-stained cells, the percentage of γ-H2AX-positive cells was calculated. As shown in Fig. 3b, approximately 43.8 and 66.7% of cells were γ-H2AX-positive at 0 and 24 h, respectively, after 1 h treatment with 5 μmol/l cisplatin. Approximately 73.3% of cells were γ-H2AX-positive immediately after treatment with 5 μmol/l β-lap for 1 h. When cells were pretreated with 5 μmol/l cisplatin for 1 h and then treated 0 and 24 h later with 5 μmol/l β-lap for 1 h, 77.7 and 92.2% of cells were γ-H2AX-positive, respectively. In summary, the percentage of γ-H2AX-positive cells increased during the 24 h after cisplatin treatment, and the combination of cisplatin and β-lap treatment with a 24-h interval was more effective than the same combination with a 0-h interval for inducing γ-H2AX foci, that is, DNA double-strand breaks.

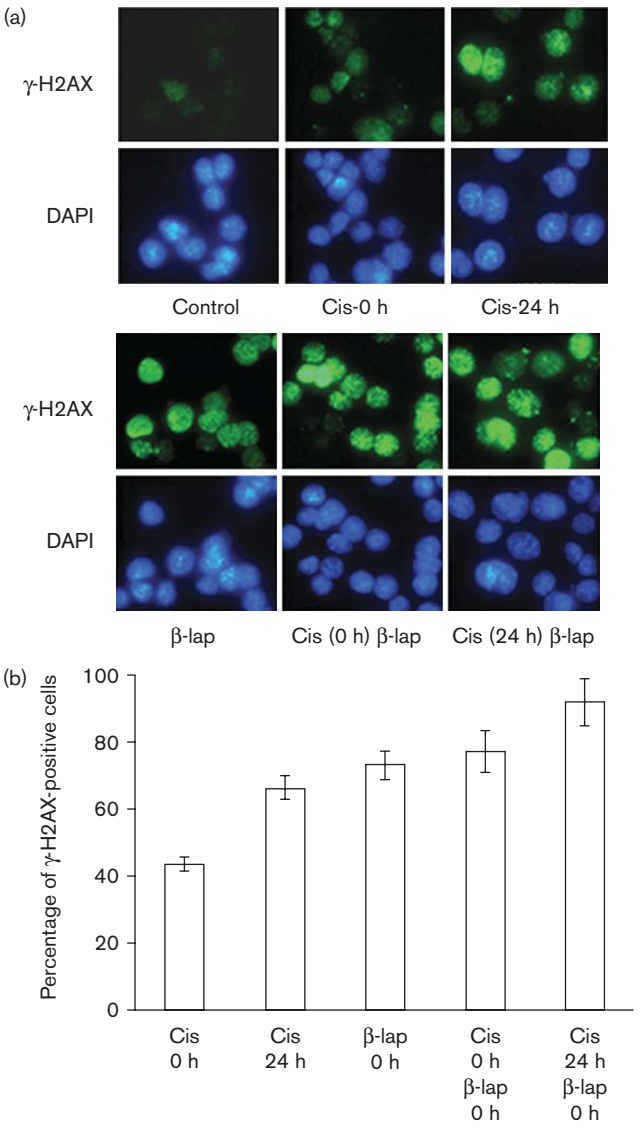
The effect of cisplatin on the expression and activity of NQO1

Figure 4a shows the expression of NQO1 in FSaII tumor cells after treatment with 2 μmol/l cisplatin for 1 h. As a reference, the nuclei of cells were stained with DAPI. The NQO1 expression in control cells was almost negligible, and a small fraction of cells expressed NQO1 immediately after the cisplatin treatment. At 12 and 24 h after cisplatin treatment, the NQO1 expression markedly increased. Figure 4b shows a representative western blotting for the NQO1 in FSaII cells treated with 2 μmol/l cisplatin for 1 h. The numbers shown above each blot are the relative intensity of the blots, obtained using the Image J computer program. The expression of NQO1 in the control FSaII cells was almost negligible, but it increased approximately two times at 24 h after the cisplatin treatment. The effect of cisplatin treatment on the NQO1 activity in FSaII tumor cells is shown in Fig. 4c. Approximately, 0.02 μmol of DCPIP was reduced/min/mg of protein before the cisplatin treatment, and the NQO1 activity increased to 0.05 μmol DCPIP/min/mg protein 24 h after the cells were treated with 5 μmol/l cisplatin for 1 h.

The role of NQO1 in the cell death caused by β-lap alone or in combination with cisplatin

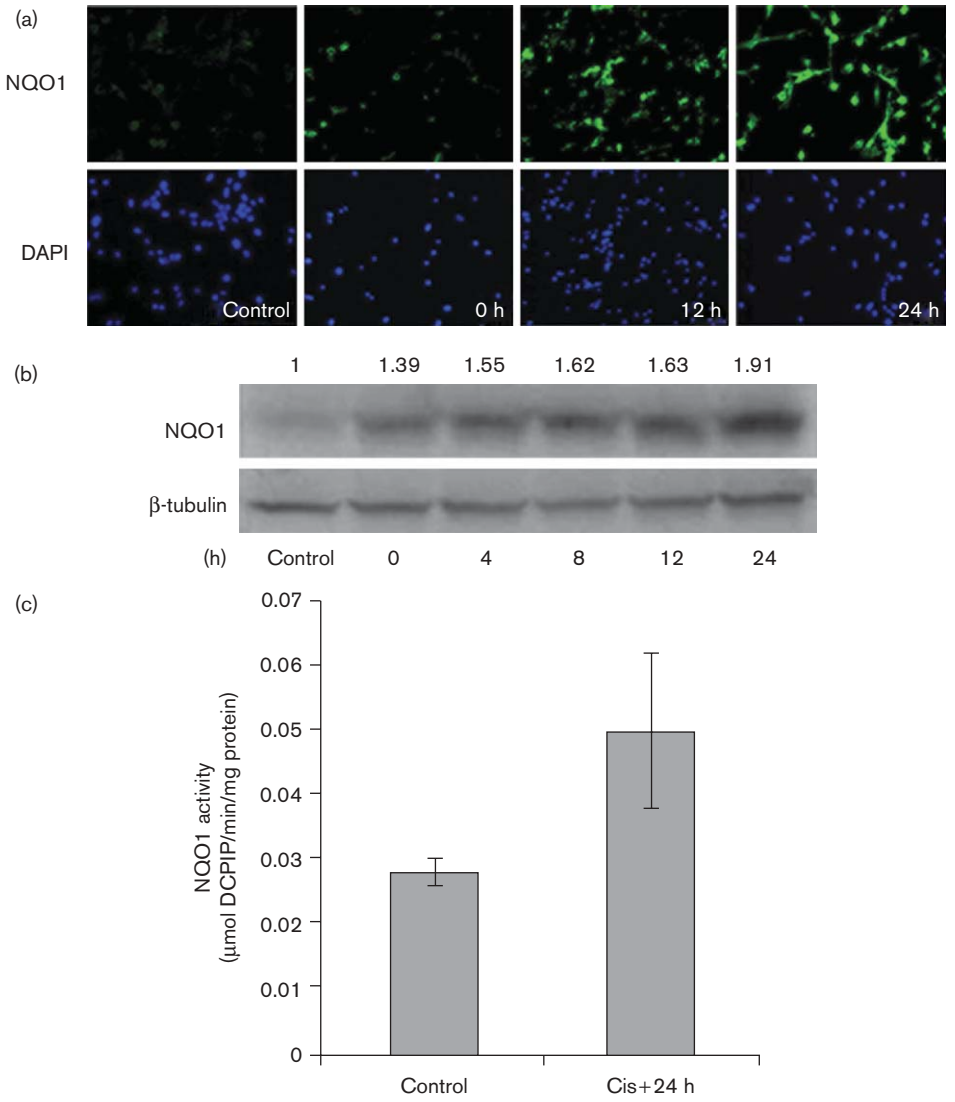
Figure 5a shows the effect of inhibition of NQO1 with dicoumarol, an inhibitor of NQO1, on the clonogenic death of FSaII tumor cells caused by β-lap alone or by a combination of cisplatin and β-lap treatments. A 4-h incubation with 5 μmol/l β-lap decreased the clonogenic cell survival to approximately 32.9 ± 0.4%. When cells were treated for 4 h with 5 μmol/l β-lap together with 50 μmol/l dicoumarol, 62.0 ± 5.0% of cells survived, which was significantly greater than the cell survival that was

Fig. 3



The formation of γ-H2X foci in FSaII tumor cells treated with 5 μmol/l cisplatin for 1 h alone and 5 μmol/l β-lapachone (β-lap) for 1 h alone or combined. (a) Confocal fluorescence microscopy examination of γ-H2X foci (upper) and DAPI staining (lower) of the cells. Control: untreated control cells. Cis-0 h, immediately after cisplatin treatment; Cis-24 h, 24 h after cisplatin treatment; β-lap, immediately after β-lap treatment; Cis (0 h) β-lap, immediately after combined treatment with cisplatin and β-lap with 0-h interval; Cis (24 h) β-lap, immediately after combined treatment with cisplatin and β-lap with 24-h interval. (b) The percentage of cells expressing γ-H2AX foci. The numbers of cells expressing γ-H2AX foci and the total numbers of cells, that is, the number of DAPI-stained nuclei, were counted and the percentage of cells expressing γ-H2AX foci was calculated. Cis 0 h, Immediately after cisplatin treatment; Cis 24 h, 24 h after cisplatin treatment; β-lap 0 h, immediately after β-lap treatment; Cis 0 h β-lap 0 h, Immediately after combined treatment with cisplatin and β-lap separated by 0-h interval; Cis 24 h β-lap 0 h, immediately after combined treatment with cisplatin and β-lap separated by 24-h interval.

Fig. 4

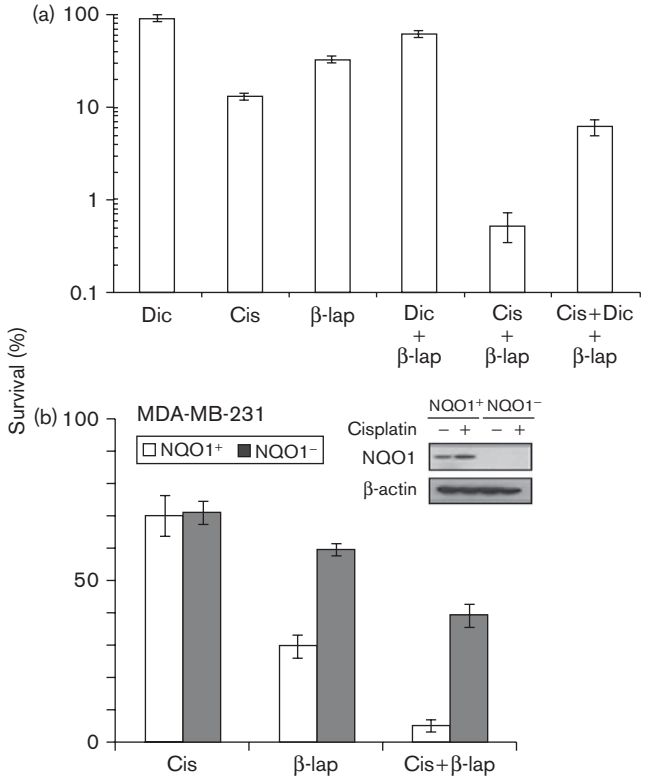


Effects of cisplatin on the NQO1 level in FSaII tumor cells. (a) Immunostaining of NQO1. Cells were incubated with 5 μmol/l of cisplatin for 1 h, washed, and incubated at 37°C under the standard culture conditions. At 0, 12, and 24 h after the cisplatin treatment, cells were incubated with anti-NQO1 antibody and then with a secondary antibody conjugated with FITC. After staining with DAPI, cells were examined with a confocal laser scanning fluorescence microscope. (b) Western blot analysis for NQO1. Cells were incubated with 5 μmol/l of cisplatin for 1 h, washed, and incubated at 37°C under the standard culture conditions. At the indicated times after cisplatin treatment, cells were dissolved in solubilizing buffer, and lysates were subjected to western blot analysis. Blots were labeled with anti-NQO1 antibody and then with a horseradish peroxidase-conjugated secondary antibody, and visualized using chemiluminescence. The numbers above the blots are the intensity of each blot relative to that for the control. (c) The effect of cisplatin on the enzymatic activity of NQO1 in FSaII tumor cells. Cells were treated with 5 μmol/l cisplatin for 1 h, incubated with complete medium for 24 h and the NQO1 activity was determined using DCPIP as substrate. Averages of four experiments with ±1 SE are shown. Cis, cisplatin.

observed after treatment with β-lap alone (32.9 ± 0.4%), showing that inhibition of NQO1 suppressed the β-lap cytotoxicity. Treating the cells with 5 μmol/l cisplatin for 1 h and then retreating 24 h later with 5 μmol/l β-lap reduced the cell survival to 0.52 ± 0.18%, showing that, in agreement with the results shown in Fig. 2, cisplatin pretreatment markedly increased cellular sensitivity to β-lap. In contrast, as much as 6.05 ± 1.17% cells survived when 50 μmol/l dicoumarol was present during the β-lap treatment after cisplatin pretreatment, indicating that the cisplatin-induced sensitization to β-lap was because of cisplatin-induced upregulation of NQO1.

Figure 5b shows the response of NQO1-positive and NQO1-negative MDA-MB-231 human breast cancer cells to the treatment with β-lap alone or to the combination of cisplatin and β-lap. The inset illustrates that NQO1 is expressed and is increased 24 h after 1 h treatment with

Fig. 5



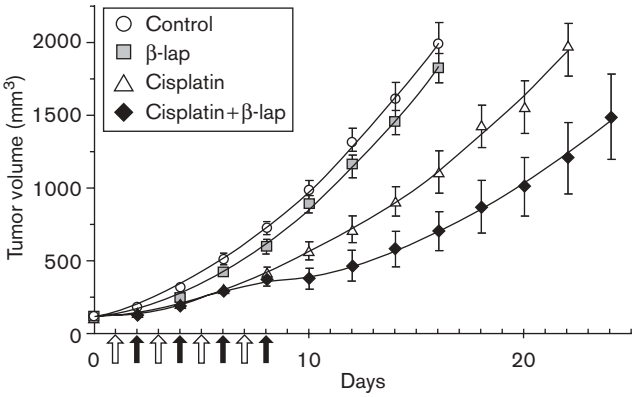
Role of NQO1 in the cell death caused by β-lapachone (β-lap) alone or in combination with cisplatin. (a) Clonogenic survival of FSaII cells after following treatment. Dic, treated with 50 μmol/l dicoumarol for 4 h; β-lap, treated with 5 μmol/l β-lap for 4 h; Dic + β-lap, treated with 50 μmol/l dicoumarol + 5 μmol/l β-lap for 4 h; Cis + β-lap, treated with 5 μmol/l cisplatin for 1 h, incubated for 24 h and treated with 5 μmol/l β-lap for 4 h; Cis + Dic + β-lap, treated with 5 μmol/l cisplatin for 1 h, incubated for 24 h and treated with 50 μmol/l dicoumarol + 5 μmol/l β-lap for 4 h. Average of six experiments ± 1 SE are shown. (b) Clonogenic survival of NQO1-positive ($NQO1^+$) and NQO1-negative ($NQO1^-$) MDA-MB-231 human breast cancer cells. Cis, treated with 5 μmol/l cisplatin for 1 h; β-lap, treated with 5 μmol/l β-lap for 1 h; Cis + β-lap, treated with 5 μmol/l cisplatin for 1 h, incubated for 24 h and retreated with 5 μmol/l β-lap for 1 h. Average of five experiment ± 1 SE are shown.

cisplatin only in the NQO1-positive, and not in the NQO1-negative, MDA-MB-231 cells. The cell death caused by 1 h incubation with 5 μmol/l β-lap alone was significantly greater in the NQO1-positive cells than in the NQO1-negative cells. Moreover, NQO1-positive cells were much more sensitive than NQO1-negative cells to the combination of 1 h treatment with 5 μmol/l cisplatin followed by 24 h after 1 h treatment with 5 μmol/l β-lap. Taken together, these results unequivocally show that NQO1 plays a cardinal role in β-lap-induced cell death and that cisplatin treatment increases cellular sensitivity to β-lap by upregulating NQO1.

Tumor growth delay by β-lap alone, cisplatin alone or the combination of the two

Figure 6 shows the effects of β-lap alone or cisplatin alone and of a combination of cisplatin and β-lap on the growth

Fig. 6



Effects of cisplatin and β-lapachone (β-lap) alone or combined on the growth of subcutaneous FSaII tumors. The C3H mice bearing about 100 mm^3 FSaII tumors in the hind legs were treated with four cycles of treatment consisting of intraperitoneal injections of 2 mg/kg cisplatin (solid arrows) and 40 mg/kg β-lap (open arrows) separated by 24-h interval. Another group of tumor-bearing mice was treated with either 2 mg/kg of cisplatin or 40 mg/kg of β-lap applied every other day for four times. The average volumes of six to nine tumors per group with ± 1 SE are shown.

of subcutaneous FSaII tumors in the hind legs of C3H mice. The volume of control tumors increased five times in 6.8 ± 0.4 days. When the host mice were treated every other day with 40 mg/kg β-lap for four total treatments or with 2 mg/kg cisplatin for four total treatments, the tumor volumes increased five times in 7.7 ± 0.4 and 10.8 ± 0.8 days, respectively. These values were 0.9 and 4.0 days longer, respectively, than the time required for the control tumors to grow five times in volume. The tumor volume increased five times in 15.4 days, that is, 8.6 days longer than that for the control tumors, when host mice were treated with a combination of cisplatin and β-lap. It can be concluded that cisplatin and β-lap reacted more than additively in suppressing tumor growth (for a more detailed analysis, see Discussion).

Discussion

In this study, pretreatment with cisplatin markedly sensitized tumor cells to subsequent β-lap treatment. Importantly, the β-lap sensitivity progressively increased and NQO1 expression/activity also increased for 24 h after cisplatin treatment. In light of the critical role of NQO1 in the β-lap-induced cell death, the cisplatin-induced sensitization to β-lap may be attributed to the cisplatin-induced upregulation of NQO1.

As shown in Fig. 1, β-lap caused clonogenic death in FSaII tumor cells *in vitro* in a dose-dependent manner. When cells were treated with cisplatin and immediately treated with β-lap, the cell death caused by the combined treatments was additive (Fig. 2a). However, the cell death was more than additive when cells were pretreated with cisplatin and treated 12–24 h later with β-lap. To further

show the combined effect of cisplatin and β-lap, cells were pretreated with 5 μmol/l cisplatin for 1 h and exposed 24 h later to graded doses of β-lap for 4 h. As shown in Fig. 2b, the survival curve for the cells pretreated with cisplatin and then treated with β-lap 24 h later was markedly steeper than that for the cells treated with β-lap alone, showing that the pretreatment with 5 μmol/l cisplatin sensitized the cells to β-lap.

The γ-H2AX foci formation, hallmark of DNA double-strand breakage, caused by β-lap treatment in the FSaII tumor cells (Fig. 3) was in good agreement with earlier reports [2,13]. The magnitude of γ-H2AX foci formation is often determined by counting the number of foci in each cell [13,30]. However, it was observed during this experiment that counting the number of foci in each cell becomes increasingly inaccurate as the number of foci increases. Therefore, for this experiment, the number of cells exhibiting more than seven foci was counted, and the percentage of γ-H2AX-positive cells was calculated using that number. As shown in Fig. 3b, the percentage of γ-H2AX-positive cells was larger in the cells pretreated with cisplatin and then treated 24 h later with β-lap than in the cells treated with cisplatin alone, β-lap alone, or treated with cisplatin and immediately retreated with β-lap.

The confocal laser scanning microscopic examination of the immunostained cells (Fig. 4a) and the western blotting study (Fig. 4b) clearly show that pretreatment with cisplatin increased the NQO1 expression in the cells over the 24 h after the treatment. As shown in Figs 2 and 3, the sensitivity of FSaII cells to β-lap increased 12–24 h after cisplatin pretreatment, particularly at 24 h after pretreatment. Importantly, inhibition of NQO1 activity with dicoumarol reduced the cell death caused by β-lap and prevented the cisplatin-induced sensitization of cells to β-lap treatment (Fig. 5). Furthermore, cisplatin-induced sensitization to β-lap was markedly greater in NQO1-positive than NQO1-negative MDA-MB-231 cells (Fig. 5b). In our earlier studies, ionizing radiation [27,28,30] or mild-heat shock [29,31] was found to upregulate NQO1 expression in cancer cells, and such an increase in NQO1 activity sensitized cancer cells to β-lap. Taken together, it is concluded that cisplatin treatment upregulates NQO1 in cancer cells, which, in turn, markedly increases the sensitivity of the cells to subsequent β-lap treatment. The activation of the NQO1 gene by various oxidative stresses and xenobiotics has been reported to be regulated by the antioxidant response element present in the cis-element gene promoter and by the transcription factor Nrf2 [35–37]. Experiments to shed light on the mechanisms by which cisplatin treatment upregulates NQO1 in cancer cells is in progress in our laboratory.

Figure 6 shows that the control tumor volume increased five times in 6.8 days. When the host mice were treated with 40 mg/kg β-lap on alternate days for four total treatments, tumor volume increased five times in 7.7 ± 0.4 days, which was 1.1-times longer than that required for the control tumors to increase by a similar proportion. When treated with 2 mg/kg cisplatin on alternate days for four total treatments, the tumor volume increased five-fold in 10.8 ± 0.8 days, which was 1.6-times longer than required for the control tumors. The combined treatment with cisplatin and β-lap, one given on alternate days for four treatments each, significantly suppressed the tumor growth so that tumor volume increased five times in 15.4 ± 1.4 days, which was 2.3-times longer than that required for the control tumors to increase by a similar proportion. If the combination of β-lap and cisplatin suppressed tumor growth in an additive manner, the tumor growth time would be delayed by 1.8-times (1.1×1.6), as opposed to 2.3-times that observed. It may then be concluded that the combined treatment with cisplatin and β-lap suppressed tumor growth in a manner greater than additive. Note that the host mice were treated with cisplatin first and then 24 h later with β-lap, repeating the cycle four times. On the basis of the results of in-vitro studies, it may be assumed that cisplatin increased the NQO1 level in the tumors, thereby potentiating the effect of β-lap against the tumor cells. In this study, β-lap solution was injected i.p. to the tumor-bearing mice. Little is known about the pharmacokinetics of β-lap in mice, but it is probable that i.p. injection may not be an effective way to deliver β-lap to target tumor cells. Interestingly, intravenous injection was more effective than i.p. injection to deliver β-lap to target tumor cells (Dr D. Boothman, November 2007, personal communication). Importantly, the NQO1 activity is higher in tumor cells than in normal cells [6,23,32–34]. It remains to be determined whether β-lap treatment preferentially destroys malignant tissues relative to normal tissues *in vivo*. It also remains to be seen whether upregulation of NQO1 by cisplatin, radiation, or heat shock also potentiates the cytotoxicity of other NQO1-directed bioreductive drugs, such as MMC, RHI, EO9, and ARH019 [14–20,22–26].

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